Otarine herpesvirus-1 (OtHV-1) is associated with high rates of urogenital carcinoma in free-ranging California sea lions (*Zalophus californianus*), but rarely considered a conservation concern in the southern hemisphere. The objective of this study was to survey free-ranging South American sea lions (*Otaria byronia*) and Peruvian fur seals (*Arctocephalus australis* unnamed subspecies) in Punta San Juan, Peru for OtHV-1 and to determine prevalence by demographic factors. Twenty-one percent (14/67) of urogenital swabs collected over three years (2011, 2014, 2015) from live pinnipeds tested positive with a pan-herpesvirus conventional PCR. Sequencing revealed 99% homology to OtHV-1 at the DNA polymerase gene. Urogenital, conjunctival, and oropharyngeal swabs collected from 136 live pinnipeds at Punta San Juan between 2011-2018 were then assayed using quantitative PCR for a segment of the OtHV-1 DNA polymerase gene. In total, 38.6% (51/132) of urogenital swabs, 5.6% (4/71) of conjunctival swabs, and 1.1% (1/90) of oropharyngeal swabs were positive for OtHV-1. Agreement in OtHV-1 detection between sampling sites was minimal to non-existent (Cohen’s Kappa=0.027-0.386). The most parsimonious multivariable logistic regression model predicting OtHV-1 detection (P < 0.0001) included species and age class, with South American sea lions (32/81, 39.5%) having a higher prevalence of OtHV-1 in urogenital swabs than Peruvian fur seals (19/51, 37.2%), and adults (46/95, 48.4%) having a higher prevalence than pups (5/37, 13.5%). In addition, female South American sea lions had higher copy numbers (median=8,819 copies/ng DNA) than males (median: 27 copies/ng DNA, P=0.012), and adult South American sea lions (median=219 copies/ng DNA) had higher copy numbers than pups (median: 3 copies/ng DNA, P=0.008). The much higher prevalence in adults compared with pups, as well as the higher sensitivity in
urogenital swabs, suggests a sexual transmission, which is similar to California sea lions. These data provide insight into dynamics of the potentially oncogenic OtHV-1 in a novel ecosystem, emphasizing the importance of continued disease surveillance in vulnerable Peruvian marine mammal populations.
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CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Otarine herpesvirus-1, a gammaherpesvirus in the family Herpesviridae, has been detected in clinically healthy free-ranging California sea lions (Zalophus californianus, CSL), and is also closely associated with high rates of metastatic urogenital carcinoma in the same species. This uniquely common wildlife neoplasia was found in 18% and 12% of subadult and adult stranded CSL in California from 1979-1994 and 2005-2015, respectively (Gulland et al., 1996, Deming et al., 2018). Thus far, OtHV-1 infection has only been reported in CSL in California, USA and the Gulf of California, Mexico, and in one South American fur seal (Otaria byronia) in Europe which likely acquired the virus in managed care (Dagleish et al., 2013, Barragan-Vargas et al., 2016). There are no reports of OtHV-1 in the southern hemisphere. Interestingly, while CSL in Mexico have similar prevalences of OtHV-1 compared with Californian animals, there are currently only isolated recent reports of urogenital carcinoma at this location (Barragan-Vargas et al., 2016, Colegrove pers. comm.). This disparity in neoplasia prevalence presents an opportunity for research examining factors other than viral infection that may play a role in development of urogenital carcinoma. However, necropsy surveillance in the Gulf of California is not nearly as extensive as California, which limits the validity of the findings. Decades of research has explored the multifactorial nature of the carcinogenesis in CSL and shown possible relationships with high levels of elevated levels of polychlorinated biphenyls in blubber, the presence of MHC class II locus Zaca-DRB.A genotype, homozygosity at the Pv11 microsatellite locus, endogenous hormones, alteration in p53, and beta-hemolytic Streptococcus infection in females in addition to OtHV-1, (Brouwer et al., 1989, de Swart et al., 1996, Johnson et al., 2006, Colegrove et al., 2009, Browning et al., 2014, Barragan-Vargas et al., 2016).
While established geographic ranges of many otariid species on the west coast of North and South America may not overlap, aberrant movement of individuals as well as unknown migratory patterns at sea may allow for transfer of infectious diseases. Juan Fernandez fur seals (*Arctocephalus philippii*), Galapagos fur seals (*Arctocephalus galapagoensis*), Galapagos sea lions (*Zalophus wollebaeki*), Guadalupe fur seals (*Arctocephalus townsendi*), and Antarctic fur seals (*Arctocephalus gazella*) may all be found aberrantly at Punta San Juan (Adkesson, pers. Comm.). Additionally, as pinnipeds occupy both marine and terrestrial environments, they occupy unique ecologic niches that have the potential to create exposure to anthropogenic stressors that affect both ecosystems, including hunting, habitat encroachment, overfishing, and pollution (Harkonen et al., 2012, Cárdenas-Alayza et al., 2016a, b). Understanding health parameters in vulnerable populations is critical to elucidating current health risks and identifying factors of conservation concern.

Punta San Juan is a Marine Protected Area in Peru that is home to key breeding rookeries for two locally endangered pinnipeds: South American sea lions (*Otaria byronia*) and Peruvian fur seals (*Arctocephalus australis* unnamed subspecies) (Cárdenas-Alayza et al., 2016a, b). Health surveys have been conducted on wildlife species at Punta San Juan since 2007, but neither pinniped has been screened for otarine herpesviruses (Jankowski et al., 2015). Necropsy surveillance at the site is very limited and only recently established, and no cases of urogenital carcinoma have been reported. Knowledge of the presence or absence of OtHV-1, as well as effects of various demographic parameters on prevalence (if present), may help to target future research into pathogens potentially affecting the health of these species.

In addition, examining potential shedding sites for the virus may better define the ideal diagnostic sample, and has the potential to identify species differences in viral shedding.
Extensive necropsy data from CSL with urogenital carcinoma have demonstrated that OtHV-1 is most often detected in organs located in close proximity to the urogenital tract, such as lumbar lymph nodes, the urinary bladder, and the cervix (Buckles et al., 2006). However, in some animals with urogenital carcinoma and a few individuals with no clinical signs, OtHV-1 DNA has been detected in the salivary glands and tonsils (Buckles et al., 2006). The virus was detected in a single pharyngeal sample in a CSL with a concurrently positive preputial swab, indicating that tissue tropism might expand outside the urogenital tract (Buckles et al., 2007). Investigating additional sampling sites will provide a better characterization of tissue distribution and potential shedding sites.

The overall goal of the research presented in this thesis was to perform surveillance of vulnerable populations of pinnipeds in Peru for herpesviruses, which have been known to have potential significant population health consequences. After OtHV-1 was detected in the samples, epidemiology of OtHV-1 was explored in these species with the goal of focusing future research and conservation efforts in these populations of South American sea lions and Peruvian fur seals in Peru. The following objectives for this thesis include:

1. Surveillance of South American sea lions and Peruvian fur seals at Punta San Juan for the presence of herpesviruses
2. Establishment of baseline prevalences for otarine herpesvirus-1 in these populations
3. Evaluation of the effects of species, sex, age class, and swabbing site on the prevalence of otarine herpesvirus-1
CHAPTER 2: LITERATURE REVIEW

Pinnipedia Taxonomy and Natural History

Pinnipeds are a monophyletic group within the order Carnivora and possess unique adaptations that allow them to occupy both terrestrial and aquatic environments. Their shared characteristics include fur, long vibrissae, nares at the tip of the rostrum, strongly reduced or absent pinnae, and the need to return to land for parturition (Jefferson et al., 2008b). However, species have different methods of land and water locomotion and vastly different social structures. Pinnipeds have a worldwide distribution and their need for coastal habitat results in frequent contact, and often clashes, with anthropogenic society.

There are 34 extant pinniped species that belong to three families: Otariidae, Phocidae, and Odobenidae. Otariids are eared seals and consist of sea lions and fur seals. They have small external pinnae, a double layer of fur for thermoregulation, smooth vibrissae, hairless pelvic limbs, and pronounced sagittal crests in adult males (Jefferson et al., 2008b). Notably, they have large foreflippers and have the ability to rotate their hind flippers forward to allow for quadruped-like agility on land. Their foreflippers are used as their primary method of propulsion in water. Sexual dimorphism is dramatic in most species, with males sometimes outweighing females by more than four times, as is the case in Steller sea lions (*Eumetopias jubatus*) (Jefferson et al., 2008a). Their social structure is polygynous and some species are social and found regularly in large groups, or rookeries (Jefferson et al., 2008).

Phocids are the earless seals, or true seals. As their name suggests, they lack pinnae, and in contrast to otariids, have beaded vibrissae, shorter muzzles, furred pelvic limbs, short fur, and an absence of a sagittal crest (Jefferson et al., 2008b). Propulsion in the water is created by
swimming in an S-shape with their hindflippers, using their foreflippers mostly to steer. On land, they are less agile than otariids and move by “galumphing”, which is a motion resembling that of an inchworm (Jefferson et al., 2008b). Compared with otariids, they spend more time in the water than on land. However, similarly to otariids, their body size sexual dimorphism may also be marked and many species aggregate on rookeries.

Odobenidae consists only of walruses (*Odobenus rosmarus*), which combine characteristics between the otariids and phocids. Like phocids, they lack external pinnae and propulse themselves with S-shaped movements in the water, but they have large foreflippers that allow them to walk on land more like otariids than phocids. With their large size, however, they are not as agile. Unique physical features include tusks in both sexes and a barely visible tail (Jefferson et al., 2008a). They live only in the high Northern Hemisphere and have a specialized environmental niche that makes them vulnerable to environmental change (Lowry et al., 2016).

This study focuses on two species in the family Otariidae: South American sea lions (*Otaria byronia*) and Peruvian fur seals (*Arctocephalus australis*), an unnamed subspecies of the South American fur seal. South American sea lions are stocky pinnipeds that are native to the coastal waters of Argentina, Brazil, Chile, the Falkland Islands, Peru, and Uruguay (Cárdenas-Alayza, et al., 2016a). They are a polygynous species, though their social structure appears to vary depending on their geographic location. While at sea, they may be in small or large groups, but they gather on larger rookeries for the breeding season (Cárdenas-Alayza et al., 2016a). In the Peruvian populations, a lek-like mating system is established where males defend territories, but females may pass between them (Soto and Trites 2011). Females reach sexual maturity at 4-5 years, and males reach maturity at 4-7 years, though they may not defend a territory until the ages of 9-11 years (Grandi et al., 2012). Males are much larger than females, reaching weights of
350 kg as compared to 170 kg females (Grandi et al., 2012). The breeding season goes from mid-December through early-February. Parturition peaks in mid-January, and most females go into estrus about 6 days after birth, leaving for their first foraging trip about 2-3 days after copulation (Campagna 1985). Pups are generally weaned at 8-10 months old, but some have been seen suckling for up to 3 years (Campagna 1985). As marine predators, they are considered generalists, and their diet consists of many different benthic and pelagic species of teleosts and invertebrates. This allows them some flexibility to adjust their diet to natural or anthropogenic changes in prey abundance, though consequences in maternal attendance to pups as a result have been documented (Soto et al., 2006).

South American fur seals have a similar geographic range, found on the coasts of Argentina, Brazil, Chile, the Falkland Islands, Peru, and Uruguay. However, morphometric and genetic data have found evidence for separate subspecies in populations in Peru and Uruguay (Oliveira et al., 2009). The name “fur seal” is misleading, as they are otariids and not phocids (seals). Males reach sexual maturity at around age 7 years, and weigh up to 160 kg, while females reach sexual maturity earlier (around 3 years) and weigh about 60 kg (Cárdenas-Alayza et al., 2016b). Males are polygynous, defending territories of 6-20 females, and territorial males often lose body mass and are underconditioned by the end of the breeding season (Majluf 1987). Their breeding season runs from October to December, with peak parturition occurring in late November (Majluf 1987). Copulation occurs about 1 week after the mothers give birth, and shortly after, the mothers leave the beach to go on foraging trips. While pups start feeding on their own at about 6-8 months, they can be seen nursing as yearlings (Majluf 1987). Their diet is limited, with just a few pelagic teleosts (mostly anchoveta) and cephalopods making up the bulk
of their diet, and as such are highly susceptible to variations in prey availability (Majluf 1987, Cárdenas-Alayza et al., 2016b).

Conservation

Eleven of the 34 extant species of pinnipeds are near threatened, vulnerable, or endangered (IUCN 2018). Anthropogenic threats to their populations encompass those from both their terrestrial and marine habitats and include hunting, conflict with fisheries, and habitat destruction (Cárdenas-Alayza et al., 2016a, b, IUCN 2018).

For many species of pinnipeds, including South American fur seals, hunting drastically decreased populations in the late 1800s and early 1900s (Harkonen et al., 2012, Cárdenas-Alayza et al., 2016b). In multiple countries including Peru, Ecuador, Australia, New Zealand, and the United States, hunting bans provided protection to many species that were almost extirpated (Trillmich et al., 1987, Gelatt et al., 2015, Hofmeyr et al., 2015, Cárdenas-Alayza et al., 2016b). While most commercial hunting for pinnipeds has ceased, severe genetic bottlenecks occurred in some of these species, including South American fur seals, that may have long-lasting fitness and population recovery consequences (Wynen et al., 2000, Oliveira et al., 2008, Stoffel et al., 2011). This has the potential to make these populations sensitive to additional environmental stressors, such as climate events or infectious diseases (Oliveira et al., 2008).

Conflict between pinnipeds and fisheries includes not only prey depletion, but also mortality from bycatch and culling due to perceptions by humans of prey competition (Pauly et al., 1987, Crespo et al., 2001, DeMaster et al., 2001, Underwood et al., 2008, Cárdenas-Alayza et al., 2016a, b, Machado et al., 2016). Variation in the predator-prey relationship results in various consequences from overfishing, which can include large-scale decreases in pinniped populations (Crespo et al., 2001, DeMaster et al., 2001). For example, the groundfish fishery in the North
Pacific caused localized depletion of key prey species, which necessitated increased energy expenditure in Steller sea lions secondary to decreased foraging efficiency (DeMaster et al., 2001). Recovery from prey reduction can unfortunately create the need, or perceived need, to limit predation by pinnipeds. In California sea lions (Zalophus californianus) and harbor seals (Phoca vitulina) in the Eastern Pacific, recovery efforts for salmonids have placed pressure on fisheries to decrease the population of pinnipeds in an effort to preserve fish populations for human consumption (DeMaster et al., 2001). In addition, stray nets can cause unintentional entanglements even after the material has been abandoned by fisheries, including in remote areas far away from initial fishing grounds (Hofmeyr et al., 2006, Gregory et al., 2009). Studies focusing on direct interactions between pinnipeds and fisheries provide evidence that pinnipeds are not actually a direct source of decreased fishing success (Machado et al., 2016). Creating site-specific guidelines for fisheries has identified populations of pinnipeds that can withstand greater fishing rates, and those that need more stringent restrictions for population conservation (Underwood et al., 2008).

Destruction or reductions of habitat is associated with adverse effects on pinniped populations due to increased proximity to humans and/or domestic animal species. This may be through direct mechanisms such as destruction of valuable breeding ground, or oil spill contamination of habitat decimating populations by interfering with pinnipeds’ ability to thermoregulate (Mearns et al., 1999). However, indirect consequences of human proximity can be equally as damaging. Declining reproductive rates in California sea lion populations in the Gulf of California occurred as exposure to human settlements increased (French et al., 2011). As human populations expand and encroach on historic pinniped rookeries, spillover of pathogens and pollutants are increasingly likely (Daszak et al., 2001, Miller et al., 2002, Faust et al., 2018).
Pollutants running off from the land cause unavoidable change to aquatic environments and are known to biomagnify in apex predators like marine mammals (Alava et al., 2012). In some cases, they can be passed directly from mother to offspring, further compromising an already delicate juvenile immune system (Reijnders et al., 1986, Greig et al., 2011). Pollutants’ ability to alter gene expression and disrupt the immune system may make species more susceptible to infectious pathogens (Brouwer et al., 1989, de Swart et al., 1996, Tabuchi et al., 2006, Niño-Torres et al., 2009).

Changes in factors such as environmental parameters, species geographic ranges, stressors, or immune responses may change what naïve infectious pathogens are present, as well as prevalence or clinical consequences of existing pathogens (Vanwormer et al., 2013). Opportunities for direct disease transmission also increase as natural habitat shrinks and pinnipeds come into contact with feral species (Vanwormer et al., 2011). Knowledge of the current infectious disease status in wildlife populations and how they may be changing is integral to understanding the health of ecosystems and conserving coastal species. Comprehensive studies on population trends and health status of populations provides a baseline through which relationships with anthropogenic stressors can be monitored and therefore potentially mitigated (Crespo et al., 2001, French et al., 2011, Greig et al., 2011).

In multiple populations of pinnipeds, infectious disease surveys have been able to define the existing status of infectious pathogens, in some cases highlighting where interspecies transmission may occur (Burek et al., 2005, Aguirre et al., 2007, Jankowski et al., 2015, Bauer et al., 2016, Bellehumeur et al., 2016). Hawai’ian monk seals (*Monachus schauinslandi*), for example, were identified to be naïve to many mammalian diseases, including morbilliviruses like phocine and canine distemper, likely because of their geographic isolation (Aguirre et al., 2007).
Based on models simulating potential future outbreaks, the decision was made to prophylactically vaccinate free-ranging Hawai’ian monk seals for morbillivirus (Baker et al., 2017). While the direct benefits of this program will be difficult to quantify, it remains an excellent example of how diligent surveillance and knowledge of infectious diseases in populations can lead to positive conservation measures.

The study species, South American sea lions and Peruvian fur seals, are vulnerable due to their dependence on localized large breeding rookeries and the large variance of prey availability in the area during El Niño events. Punta San Juan (PSJ), a marine protected area, is home to rookeries for both of these species. Both species are listed as being of least concern according to IUCN, but are federally protected by the Peruvian government (Cárdenas-Alayza 2016a, b). These species experienced large population declines due to commercial hunting in the early 1900s, overfishing, and El Niño Southern Oscillation (ENSO) events that have prevented their populations’ recovery (Majluf 1987). In a cyclical pattern, ENSO events cause warm water upwelling that alter prey location relative to pinniped rookeries, increasing the necessary time and energy spent hunting. Females have been documented spending longer times foraging and away from their pups, resulting in starvation of younger pups (Majluf 1987). In addition, with fewer nutrients in the water, the fish that the pinnipeds do consume are often of lesser caloric value, placing both mothers and pups at risk of negative energy balance (Majluf 1987). Depending on the adaptability of the species to eat different food items, this results in increased morbidity and mortality, and decreased overall reproductive success (Majluf et al., 1998). Additionally, heavy commercial fishing in the 1950s and 1960s greatly decreased the populations of anchoveta on which these species depend (Pauly and Tsukayama 1987).
PSJ has been monitored annually by a program organized and funded by the Peruvian government, the Chicago Zoological Society, and Cayetano Heredia University. Recent serologic surveys of the populations found that they were naïve to phocine and canine distemper viruses, five *Leptospira* serovars, *Brucella canis, Toxoplasma gondii, Neospora caninum*, and *Sarcocystis neurona* (Jankowski et al., 2015). They had demonstrated exposure to *Mycoplasma* (37.9%), marine *Brucella* (53.7%), and phocine herpesvirus-1 (PHV-1) (85.7%) (Jankowski et al., 2015). However, data from the same populations have since documented exposure to *Neospora caninum, Sarcocystis neurona, Toxoplasma gondii*, and several *Leptospira* spp. serovars (Adkesson, pers comm). Otarine herpesviruses have yet to be surveyed in PSJ, but are shown to be of important conservation concern in other otariid populations.

**Herpesviridae**

*Herpesviruses taxonomy*

The order *Herpesvirales* includes three families, including *Herpesviridae, Alloherpesviridae* and *Malacoherpesviridae*. Mammal, avian, and reptile viruses occur within the family *Herpesviridae*, while fish and frog viruses as well as a bivalve virus occur in the *Alloherpesviridae* and *Malacoherpesviridae* families, respectively (Davison et al., 2009). There is a great deal of variety within herpesvirus genomes, but they do have a set of distinct morphological characteristics. They are linear, enveloped, double-stranded DNA viruses contained within a T = 16 icosahedral capsid. This capsid is surrounded by the tegument, a proteinaceous matrix, which is inside the lipid envelope (Davison et al., 2009, Pellett et al., 2012). In addition to their morphological similarities, they all appear to have the ability for latent infection, contributing to their survival success. Most species are thought to have their own
species-specific herpesviruses and in general, each species of herpesvirus is well adapted to their host, while clinical disease most often occurs in immunocompromised individuals or aberrant hosts (Pellett et al., 2012). Routes of transmission include aerosol spread, sexual contact, or oral contact (Pellett et al., 2012).

There are three subfamilies of *Herpesviridae* comprising *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. These were originally divided based on antigenic cross-reactivity as well as the size and structure of their genomes, which have been supported by subsequent phylogenetic analysis (Pellett et al., 2012). In general, not without exception, alphaherpesviruses tend to establish latent infection in neurons, betaherpesviruses in monocytes, and gammaherpesviruses in lymphocytes (Pellett et al., 2012). Alphaherpesviruses may be more likely to infect a variety of cell types *in vitro* (Pellett et al., 2012). Similarly, alphaherpesviruses may infect a wider variety of species, while betaherpesviruses and gammaherpesviruses tend to have a more restricted host range (Pellett et al., 2012).

**Herpesvirus diagnosis**

Detection of herpesvirus and diagnosis of disease can be made via multiple methods including serology, histopathology, immunohistochemistry, and polymerase chain reaction (PCR). These can be further classified as disease detection (histopathology, immunohistochemistry), disease exposure (serology), and pathogen presence (PCR). All three classifications of diagnostic testing have advantages and disadvantages. Detection of antibodies reveals information about exposure and not about current presence of the virus, and histopathology and immunohistochemistry require that the virus be present in examined sections of tissue, making diagnosis more invasive and increasing the risk of false negative results (Storch 2000). PCR detects herpesviral DNA present in the sample and tends to be easier and less
expensive than the other techniques, but does not indicate the pathogen’s ability to lead to a host response (disease) (VanDevanter et al., 1996, Storch 2000).

Due to the occurrence of herpesviruses in many species, and the difficulty of virus isolation, a molecular method to detect general herpesvirus sequences is often warranted to allow for detection of new viruses. A consensus primer PCR was developed that amplifies the DNA polymerase gene in herpesviral DNA (VanDevanter et al., 1996). A region of the DNA polymerase gene was used to design a set of degenerate primers that are highly conserved across many herpesviruses. Twenty-two randomly chosen species of herpesviruses were then tested using this consensus PCR, which resulted in 21/22 yielding products that were suitable for DNA sequencing (VanDevanter et al., 1996). This method allows for a sensitive and inexpensive screening for all herpesviruses, though the relative sensitivity varies with the specific virus (VanDevanter et al., 1996). Specificity is decreased only when the assay detects non-herpesvirus sequences between the forward and reverse primer within the ~200 base pair targeted sequence.

While consensus herpesvirus PCR allows for detection of all herpesviruses and may aid in detection of previously unknown viruses, quantitative PCR (qPCR) is a more sensitive and specific method to detect specific herpesviral species (Arikawa et al., 2008). The targeted gene sequence is generally smaller (between 60 and 150 base pairs) than that for conventional PCR (Udvardi et al., 2008). qPCR not only allows for more specific detection of a viral species, but also allows for quantification of the viral copies within the sample. There are two methods for qPCR: TaqMan and SYBR green. Both have forward and reverse primers, but SYBR green dye assays require melting curve analysis and sequencing to confirm that non-specific amplification or primer-dimer formation did not occur (Arikawa et al., 2008). In the TaqMan method, there is a probe nested within the sequence to increase specificity (Niesters et al., 2001).
Non-otarine pinniped herpesviruses

A multitude of herpesviruses have been found in all three families of pinnipeds. In the current literature, there are seven phocid herpesviruses and six otarine herpesviruses (Table 1). The seven phocid herpesviruses include both alpha- and gammaherpesviruses. Phocid herpesvirus-1 (PHV-1), first described in 1985, has been best described and is known to be associated with multiple lesions (Osterhaus et al., 1985, Gulland et al. 1997, Colegrove et al., 2018). Antibodies to PHV-1 have been found all over the world in Hawai’ian monk seals, hooded seals (Cystophora cristata), harp seals (Pagophilus groenlandicus), grey seals (Halichoerus grypus), Steller sea lions, harbor seals, South American fur seals, Antarctic Weddell seals (Leptonychotes weddellii), Antarctic fur seals (Arctocephalus gazelle), Ross seals (Ommatophoca rossii), walruses, Northern fur seals (Callorhinus ursinus), California sea lions, spotted seals (Phoca largha), ribbon seals (Histriophoca fasciata), bearded seals (Erignathus barbatus), ringed seals (Pusa hispida), and crabeater seals (Lobodon carcinophagus) but may represent cross reaction to a similar herpes virus (Stenvers et al., 1992, Harder et al., 1996, Harder et al., 1997, Zarnke et al., 1997, King et al., 2001, Martina et al., 2002b, Goldstein et al., 2003, Goldstein et al., 2004, Burek et al., 2005, Zarnke et al., 2006, Aguirre et al., 2007, Himworth et al., 2010, Tryland et al., 2012, Roth et al., 2013, Jankowski et al., 2015, Bauer et al., 2016, Bellehumeur et al., 2016). Morbidity and mortality are primarily described in juvenile harbor seals and are characterized by adrenal necrosis, hepatic necrosis, nonsuppurative encephalitis, and pneumonia (Osterhaus et al., 1985, Borst et al., 1986, Gulland et al., 1997, Harder et al., 1997, Martina et al., 2002b, Goldstein et al., 2003, Himworth et al., 2010). Both horizontal and vertical transmission are proposed due to shedding in vaginal and nasal secretions, and evidence of infection in premature neonate harbor seals (Goldstein et al., 2004). Due to the
significant clinical disease caused by PHV-1 in phocids in rehabilitation centers, and the close association between feline herpesvirus-1 (FHV-1) and PHV-1, a vaccination was developed and tested in domestic cats for protection against FHV-1 (Martina et al., 2002a, Martina et al., 2003a, b). It reduced shedding of FHV-1 and FHV-1-associated weight loss, so the vaccine was subsequently tested on harbor seals (Martin et al., 2002a, Martina et al, 2003a, b). It resulted in higher specific proliferative T cell responses, but not a higher antibody titer for PHV-1 (Martina et al., 2003b).

The other described phocid herpesviruses have fewer known clinical consequences. Phocid herpesvirus-2 (PHV-2) has been detected in harbor and grey seals, though antibodies have also been found in walrus, Northern fur seal, spotted seals, ribbon seals, Steller sea lions, bearded seals, and ringed seals (Harder et al., 1996, Zarnke et al., 1997, Martina et al., 2003c). It has not been shown to cause significant disease (Zarnke et al., 2006). Phocid herpesvirus-3 (PHV-3) was first detected from Hawai’ian monk seals, and a higher proportion of animals in professional care tested positive than free-ranging animals, but no clinical signs have yet been associated with the virus (Goldstein et al., 2006). Phocid-herpesvirus-4 (PHV-4) DNA was detected via PCR from Northern elephant seals (Mirounga angustirostris) and was associated with ulcerations on the tongue, palatine mucosa, and tonsils, though it was also detected in healthy pups (Goldstein et al., 2006). DNA of phocid herpesviruses-5 and -6 (PHV-5, PHV-6) were detected in harbor seals, and no clinical associations were found (Maness et al., 2011). PHV-6 DNA was also detected in ocular samples in California sea lions, Northern elephant seals, and Pacific harbor seals, but there was no difference in prevalence in that study between animals with and without corneal lesions (Wright et al., 2015). Phocid herpesvirus-7 (PHV-7) DNA was detected in harbor seals in the Netherlands and was associated with a morbidity event of
ulcerative gingivitis and glossitis (Bodewes et al., 2015). However, it was also detected in both harbor and grey seals without signs of disease (Bodewes et al., 2015).

DNA of a gammaherpesvirus from a single Pacific walrus in professional care in Spain was detected via PCR of tonsillar tissue (Melero, et al., 2014). While lymphocytolysis and lymphoid depletion was seen histologically, the virus, termed *Odobenus rosmarus* herpesvirus, likely did not contribute to the death of the individual (Melero et al., 2014).

**Otarine herpesviruses**

There are currently six described otarine herpesviruses, all of which align most closely with gammaherpesviruses. Otarine herpesvirus-1 (OtHV-1) is well documented in CSL in California, USA and the Gulf of California, Mexico, and has been closely associated with urogenital carcinoma in the Californian population (King et al., 2002, Bowen et al., 2006) This virus is the focus of this study and will be discussed in further detail.

Otarine herpesvirus-2 (OtHV-2) was first detected and sequenced in a CSL, though very little is known about potential clinical implications or prevalence (Maness et al., 2011). One report suggested that the virus may be tied to cases of necrotizing keratitis in CSL in rehabilitation, however no clinical associations have been confirmed (Nollens et al., 2004). In Wright et al. (2015), there was no difference in prevalence of OtHV-2 in ocular samples between animals with and without ocular lesions.

Otarine herpesvirus-3 was first reported in a clinical case of esophageal ulcers and B cell lymphoblastic lymphoma in a geriatric CSL in professional care (Venn-Watson et al., 2012). Viral loads of OtHV-3 were high in the same organs that had evidence of lymphoblastic lymphoma on histopathology, creating a suspicion for association between the disease processes (Venn-Watson et al., 2012). The highest viral load was in the esophagus, which clinically had
severe ulceration. Interestingly, in humans, the most common non-genital viscera affected clinically by herpesvirus is also the esophagus (Buss et al., 1979). Following the detection of OtHV-3 in this animal, positive tissue samples were found in 10 other deceased CSL, but the copy number was much lower in those animals (Venn-Watson et al, 2012). In a buffy coat sample taken from the original case animal 8 months prior to death, OtHV-3 was not detected, suggesting it acquired the infection within 8 months prior to death. Further investigation comparing detection of OtHV-3 in buffy coats of CSL in professional care and those in rehabilitation in Northern California demonstrated that stranded animals were more likely to be positive (34.9%) than clinically healthy professionally-housed animals (12.5%). When comparing between age classes in stranded sea lions, OtHV-3 positive animals were 5.1 times more likely to be yearlings, which may suggest potential clearance or latency of the virus if adult prevalence is lower. Unlike in OtHV-1, there was no sex difference in OtHV-3 prevalence, which makes sexual transmission unlikely (and transmission unknown) (Venn-Watson et al., 2012).

As opposed to OtHV-1-3, which have all been found primarily in CSL, otarine herpesvirus-4 (OtHV-4) is found primarily in Northern fur seals (*Callorhinus ursinus*). This virus shares high homology with OtHV-1 (Cortés-Hinojosa et a., 2016). It was first reported in 2015 in an ocular swab from a single CSL (3% of study animals) with corneal lesions, though overall the virus was not significantly associated with the occurrence of lesions (Wright et al., 2015). In 2016, OtHV-4 was detected separately in vaginal swabs from Northern fur seals in Alaska (Cortes-Hinojosa et al., 2016). Using a qPCR assay targeting the DNA polymerase gene on OtHV-4, viral DNA was detected in 25% of Alaskan Northern fur seals 37% of Californian Northern fur seals with no associated clinical signs. OtHV-1 and OtHV-4 are 95% homologous
in the DNA polymerase gene and 97% homologous in the partial glycoprotein B gene. This close genetic distance suggests closely related species and is smaller than even the distance between human herpesvirus-1 and human herpesvirus-2 (Pellett et al., 2012). Interestingly, although Northern fur seals share a large part of their geographic range with CSL, they have not been diagnosed with OtHV-1-associated urogenital carcinoma. This has incited the speculation that OtHV-4 may cross-protect against OtHV-1, thereby protecting Northern fur seals against the neoplasia-associated virus (Cortes-Hinojosa et al., 2016). However, there has been a lack of extensive surveillance for OtHV-1 in Northern fur seal populations.

OtHV-5 and -6 were identified in 2018 in a single South American fur seal that was stranded in Brazil (Sacristán et al., 2018). The animal had skin ulcerations on the fore and rear flippers, though no herpesviral inclusion bodies were observed within the lesions on histopathology. On PCR, two herpesviruses were sequenced: OtHV-5 from the skin and spleen and OtHV-6 from the stomach and intestine (Sacristán et al., 2018). While pathogenic changes could not be confirmed to be associated with either virus, authors suspected that OtHV-5 was associated with the skin ulcerations (Sacristán et al., 2018). As both of these viruses have only been detected in a single animal, widespread clinical associations cannot be made.

**Otarine herpesvirus 1 and urogenital carcinoma**

Of the currently described otarine herpesviruses, OtHV-1 is most commonly associated with clinical disease, specifically metastatic urogenital carcinoma in CSL. OtHV-1 has been reported in the literature outside of CSL only in a South American fur seal with urogenital carcinoma under human care in Europe, and potentially in Steller sea lions with no associated clinical signs, though this was not confirmed (Stott et al., 2005, Dagleish et al., 2013).
History

Urogenital carcinoma in CSL was first described in 1983 in a review of 1500 marine mammal necropsies that revealed a 2.5% rate of neoplasia in examined specimens (Howard et al., 1983). In a later study, Gulland et al. (1996) described 66 cases of metastatic carcinoma with similar clinical presentations observed in CSL stranded along the central California coast between 1994 and 1997. These cases comprised 18% of the subadult and adult cases necropsied during that time period, which created alarm in the authors for the potential of an infectious disease or chemical contaminants as contributors to high neoplasia prevalence (Gulland et al., 1996). There was no seasonal pattern, and the sex distribution was skewed toward adult males (58% were male, 42% were female) (Gulland et al., 1996). Masses were noted primarily in the pelvic region, and the histologic features of the masses suggested transitional cell origin, though in many cases primary tumors could not be definitively identified (Gulland et al., 1996). In a follow up study, the carcinoma was detected in affected stranded CSL from central California in genital tissues, including the vagina, cervix, uterus, penis and prepuce (Lipscomb, et al., 2000). These tumors were epithelial in origin, and electron microscopic examination of the affected tissues demonstrated viral particles consistent with herpesviruses, suggesting association with a virus (Lipscomb et al., 2000). In five of the ten animals examined, rare eosinophilic intranuclear inclusion bodies were present in the reproductive tract and oropharyngeal tissue samples, and four were positive for a novel gammaherpesvirus via consensus herpesvirus PCR: otarine herpesvirus-1 (Lipscomb et al., 2000). These same samples were also tested negative for five different papillomaviruses via Southern blot and conventional PCR, confirming that papillomavirus was not associated with the tumors (Lipscomb et al., 2000). In a recent study
spanning from 2005 to 2015, 12.4% of necropsied CSL in central California had urogenital carcinoma, with 78% of cases in advanced-stage disease (Deming et al., 2018).

**Clinical Signs of UGC**

As most cases of urogenital carcinoma are observed in stranded CSL, clinical signs typically reflect advanced stages of neoplasia and metastasis. Classic signs include abdominal distension, emaciation, hind flipper paresis/paralysis, edema of the genital tissues and hind flippers, and penile/vaginal prolapse (Gulland et al., 1996, Colegrove et al., 2018). Close physical examination may reveal small masses or plaque-like lesions with roughening, thickening, or dulling of the urogenital mucosa (Colegrove et al., 2018). However, lack of these findings on physical exam do not rule out urogenital carcinoma. Blood work and imaging may reveal renal failure as a result of hydronephrosis secondary to ureteral obstruction by enlarged sublumbar lymph nodes, which was seen in 62% of cases from one rehabilitation center (Deming et al., 2018). Common sites of metastases that may be visible on imaging include the sublumbar lymph nodes, adrenal glands, kidneys, spleen, liver, uterus, ovaries, pancreas, and the bladder (Gulland et al., 1996).

**Pathology of Urogenital Carcinoma**

Grossly, masses are most often detected in organs located in close proximity to the urogenital tract, such as sublumbar lymph nodes, adrenal glands, kidneys, cervix, and bladder (Gulland et al., 1996, Buckles et al., 2006). The masses may be surrounding the ureter, and the urinary bladder may be distended with urine. Marked peritoneal free fluid is common in advanced cases (Gulland et al., 1996). Multifocal caseous masses may be found throughout the body, including the lungs, liver, spleen, mediastinum, thoracic and abdominal lymph nodes, and the omentum (Gulland et al., 1996). Within the urogenital tract, development of neoplastic
lesions appears to be multicentric. Dysplastic and neoplastic lesions have been found in multiple regions of the cervix, vagina, penis, prepuce, and urethra in affected animals (Colegrove et al., 2009). Of animals diagnosed with urogenital carcinoma in central California between 2005 and 2015, advanced-stage disease with metastasis was seen in 78% of cases, with metastases most common in the lung and lymph nodes (Deming et al., 2018). Hydronephrosis was seen in 62% of cases, secondary to ureteral obstruction by metastases (Deming et al., 2018). In 12% of cases in one study, metastases were found without a primary lesion (Deming et al., 2018).

Histologically, large portions of cervical, vaginal, penile, or preputial epithelium may be affected (Colegrove et al., 2018). Affected epithelium is often very thickened and dysplastic, and neoplastic cells range from polygonal to round or elongate and have moderate amounts of eosinophilic cytoplasm (Lipscomb et al., 2000, Colegrove et al., 2018). There is often a high number of mitotic figures and there may be multinucleated neoplastic cells (Colegrove et al., 2018). In larger masses, central necrosis is common (Colegrove et al., 2018). Eosinophilic intranuclear inclusion bodies are seen in some cases, but are not common and are especially rare in metastatic lesions, and therefore should not be relied upon for diagnosis (Lipscomb et al., 2000, Colegrove et al., 2018).

**Multifactorial etiology of urogenital carcinoma**

Multiple other factors may play a role in development of urogenital carcinoma in California sea lions. Exposure to contaminants, bacterial infections, genotypes, and gene expression have all been shown to have some association with development of neoplasia. Because organochlorines are known to affect gene expression in genes related to immune function, concentrations of PCBs and DDTs were measured from blubber samples from animals with and without metastatic urogenital carcinoma (de Swart et al., 1996, Ylitalo et al., 2005).
There was a significant association with higher concentrations of PCBs and the probability of mortality with carcinoma (Ylitalo et al., 2005). In 2006, Johnson et al. reported that beta-hemolytic *Streptococcus* isolated from vaginal swabs in free-ranging and stranded female CSL was significantly associated with urogenital carcinoma (Johnson et al., 2006). In addition, certain class II MHC genotypes have been known to increase susceptibility to neoplasia in humans, so the class II MHC genotypes of stranded CSL with and without urogenital carcinoma were examined (Daniilidis et al., 1997, Dorak et al., 1999, Ferrera et al., 1999, Lin et al., 2001, Bowen et al., 2005). The presence of MHC class II locus *Zaca-DRB.A* was strongly associated with an increased risk of neoplasia, with an odds ratio of 3.64 within a sampling of 27 CSL with carcinoma and 22 CSL without carcinoma (Bowen et al., 2005). However, no other associations were made with either the total number of *DRB* genes or with other loci, so the relationship requires future evaluation (Bowen et al., 2005).

In 2009, Colegrove et al. examined the expression of estrogen receptor alpha (ER a), progesterone receptor (PR), p53, and Ki67 via immunohistochemistry in twelve CSL with metastatic urogenital carcinoma, four sea lions with non-metastasized urogenital carcinoma, and six control sea lions. ERa was lower in intraepithelial lesions and absent in metastatic lesions. No significant difference between tissues was found for PR expression. Ki67 index and p53 expression increased with lesion grade and were overall significantly higher in affected tissues compared with control epithelial samples (Colegrove et al., 2009). As these genes are also known to be affected by environmental contaminants, these results suggest that endogenous hormones, alterations in p53, and environmental contaminants all may be part of the multifactorial cause of OtHV-1-associated urogenital carcinoma (Brouwer et al., 1989, de Swart et al., 1996, Colegrove et al., 2009).
The central California population of CSL has some evidence of inbreeding, and decreased heterozygosity in animals with OtHV-1-associated urogenital carcinoma (Acevedo-Whitehouse et al., 2003). In Browning et al., 2014, this inbreeding depression and homozygosity was further localized, as urogenital carcinoma was specifically and significantly associated with homozygosity at the Pv11 microsatellite locus. Immunohistochemical labelling in tissue was also present within the single Pv11 genotype. These results suggest a genetic component to OtHV-1-associated urogenital carcinoma (Browning et al., 2014).

As discussed previously, California sea lions in the Gulf of California were observed with a similar prevalence of OtHV-1 from urogenital swabs (Bowen et al., 2006, Barragan-Vargas et al., 2016). Cytology was performed on genital tracts in pups and adult females and MHC class II genotyping and OtHV-1 status were analyzed on the same group of animals (Barragan-Vargas et al., 2016). Barragan-Vargas et al. (2016) found that presence of MHC II locus Zaca DRB-D was slightly protective against squamous cell atypia, though the significance of this is questionable as this technique has not been validated and there have been no studies definitively linking squamous cell atypia and development of urogenital carcinoma. Bowen et al. in 2006 found that class II MHC diversity was significantly different between Mexican and Californian populations, suggesting genetic and geographic distance may be key differences, supporting the association between certain genotypes and OtHV-1 associated urogenital carcinoma. In 2009, the Mexican CSL population was also observed with lower organochlorine levels in blubber compared with those from animals in California (Niño-Torres et al., 2009). However, linkage of these results to clinical relevance with urogenital carcinoma is limited due to lack of necropsy data in Mexico, especially compared with central California. Further investigation into the presence of OtHV-1
and urogenital carcinoma in other otariid species may be additionally helpful in elucidating co-
factors involved in this unusually common virus-associated neoplasia.

*Epidemiology of* OtHV-1 *in California sea lions*

Epidemiologic studies have been performed using free-ranging animals captured and sampled from study sites in California, Washington, and the Gulf of California in Mexico (Buckles et al., 2007, Barragan-Vargas et al., 2016). Males were sampled primarily from Washington (with one exception from California), and females and juveniles were sampled in California at a rehabilitation center (with the exception of one female in Washington) (Buckles et al., 2007). Results of PCR on urogenital swabs revealed both a sex and age predilection, with a prevalence of OtHV-1 of 46% (26/54) in adult males, 22% (16/72) in adult females, and 5.8% (7/120) in juveniles. Sexual transmission was hypothesized based on this difference in prevalence distribution (Buckles et al., 2007). In the Gulf of California in Mexico, age differences in the prevalence of OtHV-1 detection were similar, with 33.3% of adult females and 3.5% of juveniles found to be positive via PCR of urogenital swabs (Barragan-Vargas et al., 2016).

The possibility of vertical transmission in CSL has been evaluated in one study in which 26 adult females with domoic acid toxicosis were examined along with their fetuses (Buckles et al., 2007). Nine of the animals died and their fetuses were examined in utero, and 18 of the animals survived, with their pups stillborn or dying shortly after parturition (Buckles et al., 2007). None of the late term fetuses were positive for OtHV-1, despite three of the deceased dams being positive in either a uterine, cervical, or vaginal swab (Buckles et al., 2007). In another portion of the study examining prevalence in rookeries, a single positive vaginal swab from a premature pup of a positive mother was found, though contamination from the mother’s
secretions couldn’t be ruled out (Buckles et al., 2007). While no consistent evidence of vertical transmission has been identified to date, further studies evaluating placenta, milk, and neonatal tissues from OtHV-1-positive mothers are warranted.

There is only one case to date that describes OtHV-1-associated urogenital carcinoma in a pinniped species other than CSL: a professionally managed South American fur seal in Europe (Dagleish et al., 2013). That animal was believed to have contracted the virus from other pinnipeds at the facility due to lack of previous history of OtHV-1 in free-ranging South American fur seals, though testing prior to tumor development was not performed (Dagleish et al., 2013). This example suggests that there is potential for cross-species transmission.

Tissue Distribution of OtHV-1

Several studies have compared tissue distribution of OtHV-1 in CSL with and without urogenital carcinoma. In one study evaluating the difference of tissue distribution of OtHV-1 in animals with and without confirmed carcinomatosis, animals with metastatic disease predictably had wide tissue distribution, while OtHV-1 was even detected in 86% of sublumbar lymph node samples, 50% of muscle samples, and 29% of brain samples in animals without confirmed tumor status (King et al., 2002). Another study focused on the number of positive tissues based on tumor status. The tissues most commonly positive for OtHV-1 were also the tissues most commonly affected by the neoplasia, with females having fewer positive tissue sites than males (Buckles et al., 2006). In animals with urogenital carcinoma, 78% of vaginal samples were positive along with 80% of prostate, 60% of lumbar lymph nodes in males, and 78% of lumbar lymph nodes in females (Buckles et al., 2006). The more cranial organs where OtHV-1 was detected, mostly in isolated cases, included the retropharyngeal lymph node, non-specified skeletal muscle, lung, and the trigeminal ganglion (Buckles et al., 2006). In some animals with
urogenital carcinoma and a few individuals with no clinical signs, OtHV-1 DNA was detected in salivary glands and tonsils (Buckles et al., 2006). In animals with no urogenital carcinoma, tissues positive for OtHV-1 in included the prostate, prepuce, and trigeminal ganglion (Buckles et al., 2006). Not only was OtHV-1 detected significantly less often (37.5%) in control animals without urogenital carcinoma, but positive results in control animals were found in an average of 0.1 tissues (range of 0 to 1) (Buckles et al., 2006).

While OtHV-1 has been detected in tissues throughout the body, detection via swabs in animals without urogenital carcinoma is less common. In a study exploring associations between ocular lesions in pinnipeds undergoing rehabilitation in California and infectious diseases, a single pharyngeal swab was positive in a male that also had a positive urogenital swab. Urogenital swabs were much more often positive (22.2% and 46% of females and males, respectively), and no OtHV-1 DNA was detected in conjunctival swabs, corneal tissue, or aqueous humor in another study (Buckles et al., 2007, Wright et al., 2015). As gammaherpesviruses tend to be somewhat host-specific, these tissue predilections studied in CSL may or may not be consistent in other species in which OtHV-1 is detected. Therefore, testing of multiple body sites in other species is warranted.

Table 1: A summary of herpesviruses in pinnipeds.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sub-family</th>
<th>Year Described</th>
<th>Suspected host species</th>
<th>Potentially associated clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phocid herpesvirus-1 (PHV-1)</td>
<td>Alphaherpesvirus</td>
<td>1985 (Osterhaus et al., 1985)</td>
<td>Harbor seal (Phoca vitulina)</td>
<td>Pneumonia, hepatitis, adrenal necrosis</td>
</tr>
<tr>
<td>Phocid herpesvirus-2 (PHV-2)</td>
<td>Gammaherpesvirus</td>
<td>1996 (Harder et al., 1996)</td>
<td>Harbor seal, grey seal (Martina et al., 2002b, Martina et al., 2003c, Harder et al., 1996)</td>
<td>None</td>
</tr>
</tbody>
</table>
Table 1 (contd.)

<table>
<thead>
<tr>
<th>Phocid herpesvirus-3 (PHV-3)</th>
<th>Gammaherpesvirus</th>
<th>2006 (Goldstein et al., 2006)</th>
<th>Hawaiian monk seals</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phocid herpesvirus-4 (PHV-4)</td>
<td>Gammaherpesvirus</td>
<td>2006 (Goldstein et al., 2006)</td>
<td>Northern elephant seal</td>
<td>Ulcerative lesions of skin and oral mucosa (Goldstein et al., 2006)</td>
</tr>
<tr>
<td>Phocid herpesvirus-5 (PHV-5)</td>
<td>Gammaherpesvirus</td>
<td>2011 (Maness et al., 2011)</td>
<td>Harbor seal</td>
<td>None</td>
</tr>
<tr>
<td>Phocid herpesvirus-6 (PHV-6)</td>
<td>Gammaherpesvirus</td>
<td>2011 (Maness et al., 2011)</td>
<td>Harbor seal</td>
<td>None (Wright et al., 2015)</td>
</tr>
<tr>
<td>Phocid herpesvirus-7 (PHV-7)</td>
<td>Gammaherpesvirus</td>
<td>2015 (Bodewes et al., 2015)</td>
<td>Harbor seal, grey seal</td>
<td>Ulcerative glossitis and gingivitis (Bodewes et al., 2015)</td>
</tr>
<tr>
<td><em>Odobenus rosmarus</em> herpesvirus</td>
<td>Gammaherpesvirus</td>
<td>2014 (Melero et al., 2014)</td>
<td>Pacific walrus</td>
<td>None</td>
</tr>
<tr>
<td>Otarine herpesvirus-1 (OtHV-1)</td>
<td>Gammaherpesvirus</td>
<td>2002 (King et al., 2002)</td>
<td>California sea lion</td>
<td>Association with urogenital carcinoma (Buckles et al., 2006)</td>
</tr>
<tr>
<td>Otarine herpesvirus-2 (OtHV-2)</td>
<td>Gammaherpesvirus</td>
<td>2011 (Maness et al., 2011)</td>
<td>California sea lion</td>
<td>Possible necrotizing keratitis (Nollens et al., 2004)</td>
</tr>
<tr>
<td>Otarine herpesvirus-3 (OtHV-3)</td>
<td>Gammaherpesvirus</td>
<td>2012 (Venn-Watson et al., 2012)</td>
<td>California sea lion</td>
<td>Esophageal ulcers and B cell lymphoblastic lymphoma (Venn-Watson et al., 2012)</td>
</tr>
<tr>
<td>Otarine herpesvirus-4 (OtHV-4)</td>
<td>Gammaherpesvirus</td>
<td>2015 (Wright et al., 2015)</td>
<td>California sea lion, Northern fur seal</td>
<td>None</td>
</tr>
<tr>
<td>Otarine herpesvirus-5 (OtHV-5)</td>
<td>Gammaherpesvirus</td>
<td>2018 (Sacristán et al., 2018)</td>
<td>South American fur seal</td>
<td>Ulcerative cutaneous lesions (Sacristán et al., 2018)</td>
</tr>
<tr>
<td>Otarine herpesvirus-6 (OtHV-6)</td>
<td>Gammaherpesvirus</td>
<td>2018 (Sacristán et al., 2018)</td>
<td>South American fur seal</td>
<td>None</td>
</tr>
</tbody>
</table>
CHAPTER 3: FIRST CHARACTERIZATION OF OTARINE HERPESVIRUS-1 IN FREE-RANGING SOUTH AMERICAN SEA LIONS (OTARIA BYRONIA) AND PERUVIAN FUR SEALS (ARCTOCEPHALUS AUSTRALIS): PREVALENCE AND EFFECTS OF AGE, SEX, AND SAMPLE TYPE

Abstract

Otarine herpesvirus-1 (OtHV-1) is associated with high rates of urogenital carcinoma in free-ranging California sea lions (Zalophus californianus) and until recently was reported only in the Northern Hemisphere. The objective of this study was to survey free-ranging South American sea lions (Otaria byronia) and Peruvian fur seals (Arctocephalus australis unnamed subspecies) in Punta San Juan, Peru for OtHV-1 and to determine prevalence characteristics. Twenty-one percent (14/67) of urogenital swabs collected over three years (2011, 2014, 2015) from live pinnipeds tested positive with a pan-herpesvirus conventional PCR. Sequencing revealed 99% homology to OtHV-1 at the DNA polymerase gene. Urogenital, conjunctival, and oropharyngeal swabs collected from 136 live pinnipeds at Punta San Juan between 2011-2018 were then assayed using quantitative PCR for a segment of the OtHV-1 DNA polymerase gene. In total, 38.6% (51/132) of urogenital swabs, 5.6% (4/71) of conjunctival swabs, and 1.1% (1/90) of oropharyngeal swabs were positive for OtHV-1. The most parsimonious multivariable logistic regression model predicting OtHV-1 detection (P < 0.0001) included species and age, with South American sea lions (32/81, 39.5%) having a slightly higher prevalence of OtHV-1 in urogenital swabs than Peruvian fur seals (19/51, 37.2%), and adults (46/95, 48.4%) having a higher prevalence than pups (5/37, 13.5%). In addition, female South American sea lions had higher copy numbers (median=8,819 copies/ng DNA) than males (median: 27 copies/ng DNA,
P=0.012), and adult South American sea lions (median=219 copies/ng DNA) had higher copy numbers than pups (median: 3 copies/ng DNA, P=0.008). These data provide insight into dynamics of the potentially oncogenic OtHV-1 in a novel ecosystem, emphasizing the importance of continued disease surveillance in vulnerable Peruvian marine mammal populations.

**Introduction**

Worldwide, many ecosystems have undergone unprecedented change over the past 150 years due to anthropogenic activity. Coastal marine mammal species are in the unique position of being influenced by alteration to both terrestrial and aquatic environments. South American pinniped populations have experienced declines related to hunting, habitat encroachment, overfishing, and pollution (Harkonen et al., 2012, Cardenas-Alayza et al., 2016a, b). Coastal development reduces available habitat and subsequently increases intra- and inter-specific contact between animals, a setting that creates the potential for increased disease transmission (Daszak et al., 2001, Miller et al., 2002, Faust et al., 2018). These factors may alter animal health and predispose populations to novel or introduced pathogens. Understanding current and emerging infectious diseases in these populations is paramount for understanding ecosystem health and the development of sound conservation strategies for coastal species.

The Punta San Juan marine protected area (PSJ) protects critical rookeries for two pinniped species considered endangered by the Peruvian government: the South American sea lion (*Otaria byronia*, SASL) and the Peruvian subspecies of the South American fur seal (*Arctocephalus australis*) (Cárdenas-Alayza et al., 2016a, b). The Peruvian and Northern Chilean subpopulation of the South American fur seal is recognized by the International Union for the Conservation of Nature (IUCN) as a distinct, currently unnamed, subspecies with a common
English name of the Peruvian fur seal (PFS) (Berta and Churchill 2012, Oliveira and Brownell 2014). The subspecies is classified as vulnerable to extinction by the IUCN (Cárdenas-Alayza et al., 2016b). Commercial hunting in the early 1900s decimated PFS and SASL populations. Depletion of stocks by commercial fisheries and alterations in prey availability from El Niño Southern Oscillation (ENSO) events, among other factors, have limited population recovery (Fiedler et al., 2002, Soto et al., 2006). Surveillance for some infectious diseases have been conducted in female PFS (Jankowski et al., 2015), and additional studies are ongoing in both PFS and SASL to assess the role of disease in population health.

Pinniped herpesviruses are one group of pathogens that can potentially threaten population health. There are currently six described otarine herpesviruses, all of which align closest with the subfamily *Gammherpesvirinae* within the family *Herpesviridae*. Otarine herpesvirus-1 (OtHV-1) is well documented in California sea lions (*Zalophus californianus*, CSL) and has been closely associated with development of urogenital carcinoma (King et al., 2002). OtHV-2, 3, 4, 5, and 6 have also been identified in pinniped species, but the impact of infection on health is not well understood (Nollens et al., 2004, Maness et al., 2011, Venn-Watson et al., 2012, Wright et al., 2015, Cortes-Hinojosa et al., 2016, Sacristán et al., 2018). Notably, OtHV-5 and -6 were sequenced from a stranded South American fur seal in Brazil and OtHV-5 was hypothesized to be associated with skin lesions (Sacristán et al., 2018).

OtHV-1 was found in 18% and 12% of subadult and adult stranded CSL in California from 1979-1994 and 2005-2015, respectively (Gulland et al., 1996, Deming et al., 2018). The virus is suggested to be transmitted sexually based on a higher prevalence in urogenital tissues of adult males (Buckles et al., 2007). OtHV-1 associated metastatic urogenital carcinoma is largely restricted to CSL, but has been documented in a single South American fur seal housed under
professional care in Europe (Dagleish et al., 2013). While numerous studies have linked infection with the virus to development of neoplasia in CSL, multiple other co-factors have also been suggested to play a role in carcinogenesis (Lipscomb et al., 2000, King et al., 2002). Higher levels of polychlorinated biphenyls in the blubber, the presence of MHC class II locus ZacadarDRB.A genotype, homozygosity at the Pv11 microsatellite locus, endogenous hormones, alteration in p53 expression, and beta-hemolytic *Streptococcus* infection in females have been associated with neoplasia in CSL in conjunction with OtHV-1 infection (Brouwer et al., 1989, de Swart et al., 1996, Johnson et al., 2006, Colegrove et al., 2009, Browning et al., 2014). In limited surveillance, OtHV-1 positive CSL have been identified with PCR in the Gulf of California without published reports of urogenital carcinoma, however, necropsy surveillance of stranded animals is lacking and animals with urogenital carcinoma may go undetected (Barragan-Vargas et al., 2016). Similarly, Northern fur seals (*Callorhinus ursinus*, NFS) have an overlapping geographic range with CSL, but OtHV-1-associated urogenital carcinoma has not been identified despite large-scale surveillance (Cortés-Hinojosa et al., 2016). Additional surveillance for OtHV-1 in other species and geographic locations may further elucidate the role this virus plays in development of neoplasia. The objectives of this study were to (1) survey for OtHV-1 in SASL and PFS at PSJ, (2) describe the prevalence of OtHV-1 in the populations, and (3) evaluate the effect of species, sex, age class, and sampling site on the prevalence of OtHV-1.

**Materials and Methods**

**Sample collection**

A total of 83 SASL (46 adults, 37 pups) and 53 adult PFS were included in this project. Animals had been captured in 2011 and 2014-2018 for health evaluation at PSJ, Peru (15°22’S, 75°12’W) under Peruvian permits RJ 23-2011, 024-2014, 229-2015, and 019-2016-SERNANP-
RNSIIPG. All sample collection was conducted in November, except for 2017 (February) and 2018 (April). Adult animals were anesthetized under the supervision of a veterinarian. Pups under 6 months of age were either manually restrained or anesthetized based on protocol needs for concurrent projects. A sterile cotton-tipped applicator (Cardinal Health sterile cotton tipped applicator with plastic shaft, Cardinal Health, Dublin, Ohio) was used to collected urogenital swabs (URO) from the prepuce or vulva, conjunctival swabs (CON) from the inner palpebrae, and oropharyngeal swabs (ORO) from the dorsal soft palate. All swabs were placed in microcentrifuge (Eppendorf PCR tubes, Eppendorf North America, Hauppauge, NY) tubes and promptly placed on ice. Samples were frozen within 12 hours and had been maintained at -80°C until DNA extraction.

Conventional PCR

DNA was extracted according to the manufacturer’s instructions using a commercially available extraction kit (QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA). DNA quantity and purity were assessed using a spectrophotometer (Nanodrop spectrophotometer, Thermo Scientific, Wilmington, DE). Conventional PCR was performed using a pan-herpesvirus consensus assay targeting a short (~200bp) segment of the DNA polymerase gene (Vandevanter et al., 1996). Reactions were performed using Taq Polymerase (Taq DNA Polymerase Recombinant 500 U, Invitrogen, Carlsbad, CA), 10 x PCR buffer, MgCl2, dNTP mix (10 mM dNTP mix, Invitrogen, Carlsbad, CA), and dH2O for a total of 50 µl. Five microliters of the sample from the primary PCR reaction were used in the secondary reaction, with DNA extracts from *Terrapene* herpesvirus-1 as the positive control and DNase/RNase-free water as the negative control. Following the second reaction, PCR products were visualized by gel electrophoresis using 1% agarose gel. Positive samples were treated with ExoSAP-IT (USB
Corporation, Cleveland, OH) or purified from isolated gel slices (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA) and commercially sequenced in both directions (ACGT Inc, Wheeling, IL). Sequences were compared to known sequences in GenBank using BLASTN (Blast, National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD).

**Quantitative PCR**

DNA extracts from the swabs were assayed using a TaqMan quantitative PCR (qPCR) assay validated for OtHV-1, which targets the DNA polymerase gene (Deming unpubl). Real-time qPCR was performed using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, CA), and resulting data were analyzed using Sequence Detection Software (Applied Biosystems, Carlsbad, CA). Each reaction contained 12.5µl of 20x TaqMan Platinum PCR Supermix-UDG with ROX (TaqMan Platinum PCR Supermix-UDG with ROX, Invitrogen, Carlsbad, CA), 1.25µl 20x TaqMan primer-probe, 2.5µl of DNA extract, and water to a final volume of 25µl. All reactions, including positive and negative controls, were run in triplicate. Cycling parameters were as follows: 1 cycle at 50°C for 2 min followed by 95°C for 10 min, then 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds, then a final cycle at 72°C for 10 min. Positive samples were quantified using a seven-point OtHV-1 plasmid-based standard curve from $10^1$ – $10^7$ target gene copies per reaction. Resulting OtHV-1 copy numbers were then standardized based on the concentration of DNA in each sample. Final results are reported as OtHV-1 copy numbers per ng of DNA.

**Statistical analysis**

Prevalence data

Prevalences of OtHV-1 in URO, CON, and ORO swabs were calculated. Continuous variables (viral copy number) were tested for normality using the Shapiro-Wilk test. Descriptive
statistics including median, 10-90% percentiles, and minimum and maximum were tabulated. Multivariable logistic regression models were built with herpesvirus qPCR detection status (positive or negative) as the outcome variable and the categorical predictor variables of species, age class, sex, and year sampled. All models were fitted using the glm function in R studio version 1.0.136 at an alpha value of 0.05 (R Core Team 2018). An information theoretic approach was used to determine which model from the candidate set performed best using the AICcmodavg package in R studio (Burnham and Anderson 1998, Mazerolle et al., 2015). Odds ratios (OR) were calculated from the coefficients of the highest-ranking model. The Mann-Whitney U test was used to determine differences in copy number between categorical variables significant or close to significant (P < 0.1) in the logistic regression model using commercially available software (IBM SPSS Statistics 24.0, IBM, Chicago, IL).

Agreement analysis

Agreement between qPCR results from different body tissues (CON, URO, ORO) was assessed using Cohen’s kappa. Interpretation of the kappa statistic was performed using previously established criteria for no agreement (0 – 0.2), minimal (0.21 – 0.39), weak (0.4 – 0.59), moderate (0.6 – 0.79), strong (0.8 – 0.9), and almost perfect (>0.9) agreement (McHugh et al., 2012).

Results

Sample population

A total of 136 animals were sampled over six years; the distributions by year, species, sex, age class, and sample type are provided in Table 2. There were 132 URO swabs, 71 CON swabs, and 90 ORO swabs available for analysis, as not every animal had each location sampled.

Prevalence data
Conventional PCR

Conventional PCR was performed on 67 URO swabs (22 adult SASL, 45 adult PFS) from the years 2011, 2014, and 2015 to screen for herpesvirus. A total prevalence of 20.9% (14/67) was found, with 5% (1/22) of SASL positive and 29% (13/45) PFS positive. Sequencing revealed 99% homology to OtHV-1 in a PFS URO sample.

Quantitative PCR

Urogenital swabs had the highest prevalence of OtHV-1 (51/132, 38.6%; 95% CI: 30.3 – 47.5%), followed by CON swabs (4/71, 5.6%; 95% CI: 1.6 – 13.8%) and ORO swabs (1/90, 1.1%; 0 – 6%). Of the positive CON swabs, all four were from adult female PFS. One animal was also positive on URO and ORO swabs, one was also positive on a URO swab and negative on an ORO swab, and two were negative on both URO and ORO swabs. The single positive ORO swab was from a female PFS that was also positive on URO and CON swabs. Of the 21 multivariable models tested, the model that explained the greatest amount of variation in OtHV-1 prevalence included species and age class ($\chi^2$: 20.7516, df = 2, P < 0.0001) (Table 3). The odds of OtHV-1 detection in an adult was 10 times greater than for a pup (OR: 10.16; 95% CI: 3.31 – 31.18) (Table 4). The odds of OtHV-1 in a SASL were 2.7 times greater than for a PFS (OR: 2.7; 95% CI: 1.16 – 6.25) (Table 4).

The median copy number of positive URO samples was 250 copies/ng DNA (10-90th percentile: 2 – 245,608 copies/ng DNA; min-max: 0.2 – 3,055,050 copies/ng DNA). The median copy number in the 4 positive CON swabs was 9 copies/ng DNA (CI: 0 – 72 copies/ng DNA; min-max: 3 – 69 copies/ng DNA). The copy number of the single positive ORO swab was 3 copies/ng DNA. Viral copy number in positive URO samples was not significantly different (P = 0.167) between SASL (median: 94 copies/ng DNA) and PFS (median: 1,370 copies/ng DNA).
Viral copy number in adults was not significantly different between sexes in PFS (P = 0.165; median for males: 7,854 copies/ng DNA, females: 1,168 copies/ng DNA), but was significantly different for SASL (P = 0.012), where adult females (median: 8,726 copies/ng DNA; n = 17 positive samples) had a higher viral copy number than adult males (median: 26 copies/ng DNA; n = 10 positive samples). In SASL, positive adults (median: 219 copies/ng DNA; n = 27 positive samples) had a statistically higher viral copy number than pups (median: 3 copies/ng DNA; n = 5 positive samples) (P = 0.008).

**Agreement analysis**

Ninety animals had multiple sample types available (67 from all three sites, 20 from URO and ORO, and 3 from CON and ORO). There was poor agreement between URO and CON swabs (Cohen’s kappa = 0.033), between URO and ORO swabs (Cohen’s kappa = 0.027), and between CON and ORO swabs (Cohen’s kappa = 0.386).

**Discussion**

This study is the first to detect OtHV-1 in wild pinnipeds in the Southern Hemisphere. Prevalences were similar to or greater than those observed in CSL from both the Pacific coast of California and the Gulf of California, Mexico. The prevalence of OtHV-1 in adult males in the present study was 47% and 41.7% in SASL and PFS, respectively, compared with 46% in CSL from the California coast (Buckles et al., 2007). The prevalence in adult females was 73.9% in SASL and 35.9% in PFS in the present study, compared with 22% and 33% in CSL in the United States (Buckles et al., 2007) and Mexico (Barragan-Vargas et al., 2016), respectively. Finally, the prevalence in pups was 13.5% in SASL in the present study, compared with 3.5% in CSL in Mexico (Barragan-Vargas et al., 2016). These values are comparable with the exception of the high prevalence in adult female SASL.
The disparity between the results of the conventional and qPCR results are unsurprising given the expected increased sensitivity and specificity of qPCR. As the conventional PCR was a pan-herpesvirus assay, positive results may indicate the presence of herpesviruses other than OtHV-1. This would cause some samples to be positive on conventional PCR that were not positive on qPCR, which was seen in this study. In this study, the conventional PCR was used to screen the overall population for the presence of herpesviruses, which allowed for sequencing to detect currently known herpesviruses. The goal of the study was to determine prevalence specifically of OtHV-1, which was best determined using the more sensitive and specific qPCR assay (Deming unpubl).

Sexual transmission of OtHV-1 is thought to occur in CSL, which would be consistent with the higher prevalence and copy number found in the adult PFS and SASL in this study, as well as the higher prevalence of positive URO swabs compared to other sample types (Buckles et al., 2007). However, the higher copy number in female SASL compared to males, though not significant in the model, may challenge this notion. With sexual transmission, prevalence in adult males might be expected to be higher due to a single male mating with multiple females. A number of confounding variables in the present study makes this assessment difficult and warrants further investigation. While both male and female PFS were sampled during their breeding seasons (October - December), only female SASL were sampled during their breeding season (January - March) (Cárdenas-Alayza et al., 2016a, b). Male SASL were opportunistically sampled in November, which is outside their normal breeding season and may confound comparisons with sampling of females during their breeding season due to potential increased shedding during times of physiological stress. Furthermore, although the adult male SASL in this study were presumed sexually mature, they were smaller and younger males that were still likely
too small to defend a territory and thus less likely to have mated with multiple females. Adult female SASL were also only sampled during 2017 and 2018, when no PFS or adult male SASL were sampled, making direct comparisons between sex prevalences difficult to evaluate. Additionally, preputial swabbing technique may have been more effective starting in 2016 due to changes in protocol technique that included exteriorization of the penis from the prepuce. This may explain some interannual variation among males. However, sample year was not significant in the multivariable model, so either sampling techniques were consistent and prevalence did not change, or prevalence actually decreased over time in concordance with increased diagnostic sensitivity.

SASL had a significantly higher prevalence of OtHV-1 compared with PFS in this study. Whether this is biologically significant is unknown, but comparing these results to two similar species in the Northern hemisphere offers directions for future research. CSL and NFS are sympatric, and though copulation has been observed between the species, OtHV-1 associated urogenital carcinoma has not been reported in NFS. While extensive surveillance for the presence of OtHV-1 has not been performed yet in NFS, it has been proposed that neoplasia fails to develop in NFS because they are frequently detected with OtHV-4, which may be providing cross-protection (Cortes-Hinojosa et al., 2016). While the virus detected in this study was homologous to OtHV-1 at the DNA polymerase gene, there is a possibility that analysis of the sequenced virus may differ slightly from the OtHV-1 found in CSL at other gene sites. This genomic analysis may be helpful in phylogenetic analysis and in better understanding differences in species and geographic predilections for development of neoplasia.

It is not unexpected that the prevalence in SASL pups was significantly lower than that in adults. Given the significantly lower viral copy number in pups compared to adults, it is possible
that viral DNA was present from the dam. In one study examining the possibility of vertical transmission CSL, a single premature pup (2.6% of sampled animals) was positive for OtHV-1, which authors concluded indicates potential for perinatal transmission in a small number of pups (Buckles et al., 2007). Most pups in this project were several weeks to a few months old based on weight and morphometric data. Future sampling of pups of varying ages may help to clarify prevalence in juvenile SASL.

Thus far, no urogenital carcinoma has been documented in any wild pinniped in Peru, however, available necropsy data and surveillance programs are very limited. Mortality monitoring programs have been established recently at PSJ, but necropsy data is limited thus far. A previous case report documented OtHV-1 and urogenital carcinoma in a South American fur seal under professional care; however, this animal was housed in Europe and it was suspected to have contracted the virus from a CSL in the same facility (Dagleish et al., 2013). If urogenital carcinoma is not found in populations at PSJ, data from the present study may provide a useful resource for researchers studying co-factors of urogenital carcinoma in CSL in California. Exposure to organic environmental contaminants (polychlorinated biphenyls, organochlorine pesticides, and polybrominated diphenyl ethers) at Punta San Juan was documented to be at low levels not associated with detrimental health effects in Humboldt penguins (*Spheniscus humboldti*), suggesting that environmental toxins are not a present concern in the sympatric pinniped species (Adkesson et al., 2018).

Previously, CSL have been assumed to be the natural host for OtHV-1, but the results of this study suggest that the natural distribution of the virus may be broader than what has been previously recognized. Herpesviruses generally cause minimal clinical disease in their host species, but can result in severe disease in aberrant hosts (Ostrowski et al., 1998). Further
phylogenetic analysis of the OtHV-1 detected in this study, improved necropsy surveillance of pinnipeds in Peru to determine the prevalence of urogenital carcinoma, and surveillance for other otarine herpesviruses in these species are all warranted to better understand the epidemiology this virus and guide conservation programs.

Table 2. Prevalence of otarine herpesvirus-1 (OtHV-1) in urogenital, conjunctival, and oropharyngeal swabs taken from adult South American sea lions (*Otaria byronia*) and Peruvian fur seals (*Arctocephalus australis* unnamed subspecies) of both sexes over in 2011 and 2014-2018 in Punta San Juan, Peru.

<table>
<thead>
<tr>
<th>Year</th>
<th>Speciesa</th>
<th>Sex</th>
<th>Age class</th>
<th>Urogenital swabs</th>
<th>Conjunctival swabs</th>
<th>Oropharyngeal swabs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2011</td>
<td>PFS</td>
<td>Female</td>
<td>Adult</td>
<td>31</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>SASL</td>
<td>Male</td>
<td>Adult</td>
<td>7</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>2014</td>
<td>PFS</td>
<td>Male</td>
<td>Adult</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>SASL</td>
<td>Male</td>
<td>Adult</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>2015</td>
<td>PFS</td>
<td>Female</td>
<td>Adult</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>SASL</td>
<td>Male</td>
<td>Adult</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>2016</td>
<td>PFS</td>
<td>Male</td>
<td>Adult</td>
<td>6</td>
<td>N/A</td>
<td>6</td>
</tr>
<tr>
<td>2017</td>
<td>SASL</td>
<td>Female</td>
<td>Adult</td>
<td>15</td>
<td>N/A</td>
<td>14</td>
</tr>
<tr>
<td>2018</td>
<td>SASL</td>
<td>Female</td>
<td>Adult</td>
<td>8</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>SASL</td>
<td>Female</td>
<td>Pup</td>
<td>11</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>SASL</td>
<td>Male</td>
<td>Pup</td>
<td>25</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

aPFS = Peruvian fur seal (*Arctocephalus australis*), SASL = South American sea lion (*Otaria byronia*)
Table 3. AICc ranking of the multivariable logistic models relating categorical variables of 132 pinnipeds sampled at Punta San Juan, Peru in 2011 and 2014-2018. Included is the number of parameters (K), AICc, delta AICC, Akaike weight (\( w_i \)), cumulative Akaike weights (\( \Sigma w_i \)), and -log likelihood (-2LL).

<table>
<thead>
<tr>
<th>Model</th>
<th>K</th>
<th>AICc</th>
<th>( \Delta \text{AICc} )</th>
<th>( w_i )</th>
<th>( \Sigma w_i )</th>
<th>-2LL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species + Age</td>
<td>3</td>
<td>161.55</td>
<td>0</td>
<td>0.5</td>
<td>0.5</td>
<td>-77.68</td>
</tr>
<tr>
<td>Species + Age + Sex</td>
<td>4</td>
<td>163.06</td>
<td>1.51</td>
<td>0.23</td>
<td>0.73</td>
<td>-77.37</td>
</tr>
<tr>
<td>Age</td>
<td>2</td>
<td>165</td>
<td>3.45</td>
<td>0.09</td>
<td>0.82</td>
<td>-80.45</td>
</tr>
<tr>
<td>Year + Age</td>
<td>7</td>
<td>166.19</td>
<td>4.64</td>
<td>0.05</td>
<td>0.87</td>
<td>-75.64</td>
</tr>
<tr>
<td>Global</td>
<td>12</td>
<td>168.98</td>
<td>7.43</td>
<td>0.01</td>
<td>0.99</td>
<td>-75.35</td>
</tr>
</tbody>
</table>

Table 4. Parameter estimates and odds ratios (OR) for the best multivariable logistic regression model as determined by AIC for categorical variables related to 132 pinnipeds sampled at Punta San Juan, Peru from 2011 and 2014-2018.

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Estimates</th>
<th>SE</th>
<th>OR</th>
<th>OR 2.5%</th>
<th>OR 97.5%</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.856</td>
<td>0.481</td>
<td></td>
<td></td>
<td></td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Species: <em>Arctocephalus</em></td>
<td>-0.984</td>
<td>0.424</td>
<td>0.37</td>
<td>0.16</td>
<td>0.86</td>
<td>P = 0.02</td>
</tr>
<tr>
<td>Age class: Adult</td>
<td>2.319</td>
<td>0.572</td>
<td>10.16</td>
<td>3.31</td>
<td>31.18</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>
CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Infectious disease surveillance is crucial to better understanding the potential vulnerability of wildlife populations. This study expands the knowledge base not only for the infectious disease status of South American sea lions and Peruvian fur seals in Peru, but also for OtHV-1. These data represent the first detection of OtHV-1 in the southern hemisphere, expanding the known geographic and host range of the virus.

At Punta San Juan, future studies further describing the epidemiology of OtHV-1 will require more extensive sampling. Thus far, urogenital swabs have been determined to be the best sampling site for OtHV-1, and should include swabbing in the vulva for females, and inside the prepuce for males. In order to evaluate yearly trends, samples from each demographic group (adult females, adult males, juveniles) should be collected from both species in each year that sampling occurs. This will allow for direct comparisons over time that may reveal decreasing, increasing, or waxing and waning prevalences. Similarly, due to size constraints and safety considerations, adult male sea lions actively defending territories were not sampled. If OtHV-1 is sexually transmitted, as in CSL, males not actively defending territories may be expected to have a lower OtHV-1 prevalence than those that are because they would be less sexually active.

Sampling during varying seasons will allow evaluation of whether changes in the physiologic stress associated with breeding, parturition, and lactation may influence shedding. For South American sea lions, this will mean sampling prior to December for pre-breeding season, and between February and April for the breeding season at Punta San Juan. Females will pup anywhere from the end of December to the beginning of February, and come into estrus less than a week after parturition (Cárdenas-Alayza et al., 2016a). Pups are not weaned until 8-10
months of age, so the time when females are least likely to be lactating will be October or November. The breeding season for Peruvian fur seals is earlier, with most pups born from mid-November to mid-December (Majluf 1987). Breeding usually occurs within about a week of parturition, so pre-breeding sampling should occur in August or September, and breeding season sampling should occur from November to January.

The data from the present study demonstrates that OtHV-1 has a wider host range than previously thought. Additional surveillance of otariids in the Eastern Pacific, such as Northern fur seals and Steller sea lions, is warranted to better understand the distribution of this and other viruses in pinniped populations. Other species that are more likely to come in to contact with South American sea lions and Peruvian fur seals that should be surveyed include the Juan Fernández fur seal, Galapagos fur seal, Galapagos sea lion, Guadalupe fur seal, and the Antarctic fur seal.

The virus sequenced at Punta San Juan was sequenced at the DNA polymerase gene. It is possible that the OtHV-1 detected at Punta San Juan is different from the OtHV-1 in CSL. It is recommended that future studies compare homology of the DNA detected at this site to OtHV-1 from California sea lions at other gene sites. Once surveillance from a wider variety of species has been performed at multiple gene sites, phylogeny can be studied to determine how closely related otarine herpesviruses in geographically-overlapping species are, and what genetic differences may account for differences in clinical presentation.

While this research detected the presence of OtHV-1 DNA, qPCR is unable to determine whether the pathogen is causing clinical disease. As such, no clinical inferences can yet be extrapolated from these data. Establishment of a thorough necropsy surveillance system is essential to increasing understanding of the overall health status of these populations, and will
help to explore whether OtHV-1-associated urogenital carcinoma occurs at this site. Close examination for the presence of urogenital ulcers, urogenital swab collection, and collection of all organs for histopathology will aid in detection of pathology, even if a veterinarian cannot perform necropsies themselves. In addition, sampling and testing of milk, placental fluids, and any aborted fetuses or neonatal pups will help to evaluate if vertical transmission occurs.

As global climate shifts and ranges of prey and predator species shift, and as anthropogenic pressure on marine ecosystems increases, knowledge of the epidemiology and clinical repercussions of infectious pathogens in pinniped species is essential to future conservation efforts.
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